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Effects of the harmful diatom *Chaetoceros concavicornis* on respiration of rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: *Chaetoceros concavicornis* and *C. convolutus* are harmful diatom species which commonly occur in many temperate coastal marine waters and which can cause finfish mortalities when present at concentrations as low as 5 cells ml^{-1} of seawater. Death of finfish, including salmonids, by these harmful *Chaetoceros* spp. has been suggested to be caused by: (1) microbial infections of damaged gill tissue; (2) hemorrhage of gill capillaries; or (3) suffocation from excess mucus production at the sites of penetration of the gills by the spines. Our data indicate that the barbed spines of these diatoms damage the physical integrity of the respiratory epithelium of rainbow trout *Oncorhynchus mykiss*. The trout responds to this physical impairment of gill tissue by producing excessive amounts of mucus. The accumulation of this mucus on and between the secondary lamellae inhibits the gill function of O_2 uptake (and likely metabolic waste products release). The hypoxic conditions which result within the fish then cause a cascade of events, including anaerobic metabolism. Trout mortalities occur coincident with severe decreases in O_2 concentration and pH as well as increases in lactate and glucose concentrations of the arterial blood.

INTRODUCTION

Chaetoceros concavicornis and *C. convolutus* are harmful diatom species which commonly occur in many temperate coastal marine waters, including those of British Columbia (Albright et al. in press), and which can cause finfish mortalities when they are present in sufficient concentrations. As few as 5 cells of these harmful *Chaetoceros* spp. per ml of seawater can kill salmonids (Bell et al. 1974). And, because harmful *Chaetoceros* spp. which exceed these concentrations often occur in the surface marine waters of coastal British Columbia (Albright et al. in press), many penned salmon have been killed by these phytoplankters (Bell et al. 1974, Kennedy et al. 1976, Brett et al. 1978). These chain-forming, 'bullet-shaped' cells have hollow spines (setae) extending from their corners. The spines are studded with much smaller spines (spinules) along their length. Apparently, the spines break off and penetrate the (fish) gill membranes butt end first. The spinules are directed toward the tip of the spine, thus preventing the setae from coming out (Gaines & Taylor 1986).

Death of finfish by these harmful *Chaetoceros* spp. has been suggested to be caused by: (1) microbial infections of damaged gill tissue; (2) hemorrhage of gill capillaries; or (3) suffocation from excess mucus production at the sites of penetration of the gills by the spines (Bell 1961).

Within as little as 48 h of the onset of a harmful *Chaetoceros* spp. bloom, mortalities of penned salmonids can occur. Because of this very short period of time between the appearance of harmful concentrations of diatoms and the onset of mortalities, the latter 2 of the above listed possibilities appear to be the most likely causes of death. If this is so, the impairment of gill function in salmonids affected by the harmful *Chaetoceros* spp. should cause significant changes in the fishes' oxygen transport system. This study was therefore initiated to study the effects of harmful concentrations of *C. concavicornis* on the oxygen transport system, such as changes of gill lamellar structure, arterial blood oxygen tension, ventilation volume and frequency and pH values of arterial blood and erythrocytes of rainbow trout *Oncorhynchus mykiss*.

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MATERIALS AND METHODS

Fish. All experiments were carried out using 180 to 336 g rainbow trout *Oncorhynchus mykiss*, which were obtained from a commercial trout farm. All fish were transported to Simon Fraser University and placed in 20‰ salinity water at least 15 d prior to cannulation. The oxygen concentrations, pH values and temperatures of the dilute seawater in which the fish were placed varied from 8.3 to 9.7 mg ml^{-1}, 7.2 to 8.0 units and 14 to 16°C respectively. Feeding was discontinued 7 d prior to cannulation.

Anesthesia and surgical procedures. While held immobile in a net, each fish was anesthetized by immersion in neutralized tricaine methanesulfonate (MS 222, 0.1 g l^{-1}) for ca 4 min. Following this treatment, the gills were perfused with oxygenated water containing a small amount of MS 222 (0.05 g l^{-1}) by means of a recirculating pump. Throughout the subsequent surgical procedures, cooling the recirculating water to 5–6°C greatly extended the period for which the fish could be maintained under anesthesia.

While under anesthetic the fish were cannulated at the following sites:

(1) Buccal cavity. One of the nasal openings was enlarged using a no. 16 needle (Saunders 1962). Eighty cm of PE 90 polyethylene tubing was immediately passed through this hole. The end of the tubing was then heat-flared to anchor it on the inside of the mouth and cotton thread was wrapped around the tubing where it emerged from the nasal opening to prevent inward movement of the cannula.

(2) Opercular cavity. A hole was punched in the centre of one operculum with a no. 16 needle, and 80 cm of heat-flared, PE 90 polyethylene tubing was anchored in the hole as described above.

(3) Dorsal aorta. This vessel was cannulated using a modification of the method of Soivio et al. (1975). The cannulas were prepared from PE 50 polyethylene tubing. A steel wire (0.5 mm diameter) with the tip filed to a sharp point was inserted in the cannula. Before the operation the cannula was filled with Na-heparinate (Sigma), 200 IU ml^{-1} in isotonic (1%) NaCl solution. The tip of the steel wire and cannula were pushed through one of the nasal openings and then inserted into the blood vessel at the point between the first and second gill arches. Following insertion, the cannula was then pushed into the aorta until blood exited from the distal end of the cannula. The steel wire was then pulled out while the cannula was held in place in the aorta. The nearer end of the cannula was then pulled out through one of the nasal openings and fixed on the dorsal fin. The end was stoppered with a no. 23 ½ gauge needle and a 1 ml disposable syringe.

Experimental procedure. Following cannulation, each of 14 fish was placed in a metabolic chamber described by Maren et al. (1968). The chambers were then placed in four 75 × 60 × 22 cm black boxes: 8 chambers in the first 2 boxes containing treated fish , 6 in the other 2 boxes containing untreated control fish. Seawater of 20‰ salinity was recirculated through each chamber; the seawater was changed once each day. The pH and temperature of the recirculated waters varied between 7.3 and 8.0 units and 14 and 16°C respectively. The oxygen partial pressure was > 130 mm Hg during the course of the experiments. Following cannulation, the fish were allowed to recover for 48 h in the respiration chambers. During this recovery time the aortic cannulas were flushed once daily with the heparized saline.

Forty-eight hours after cannulation, *Chaetocerous concavicornis* was added to the sea water of the first 2 boxes at a concentration of ca 50 cells ml^{-1}. The fish were sampled for all experimental parameters immediately prior to exposure to *C. concavicornis*.

Analytic techniques. Arterial blood pH was measured using a Radiometer G 2971 G2 electrode in a thermostated cuvette and recorded using a Radiometer PHM 73 meter. Arterial blood oxygen tension (Pa_{O_2}), inspired water oxygen tension (Pi_{O_2}), and expired water oxygen tension (Pe_{O_2}) were measured using a Radiometer E 5046/0 electrode in a thermostated cuvette and recorded using a PHM 73 meter. The meter was calibrated prior to each P_{O_2} measurement. The electrode was exposed to humidified N_2 prior to the determination of Pa_{O_2}, Pi_{O_2} and Pe_{O_2} to improve response time and decrease hysteresis, as recommended by Moran et al. (1966).

Haematocrit values. These were determined by the microcapillary method.

Plasma lactate and glucose concentrations. These were determined spectrophotometrically using Sigma diagnostic kits.

Light and scanning electron microscopy. At the end of each experiment, branchial arches were removed from randomly selected treated and control trout and washed in a 1.1% saline solution. Parts of these washed gills were then fixed in Davidson's solution, dehydrated in ethanol, impregnated with wax, sectioned, stained with hemotoxylin and eosin and examined by light microscopy. Parts of the gills were fixed in glutaraldehyde, post-fixed in 1% OsO_4, dehydrated in ethanol and amyl acetate, critical-point dried and then coated with gold. These samples were examined using an Autoscan scanning electron microscope.

Determination of oxygen consumption. After the respiratory chamber was sealed with rubber stoppers, water within the chamber was sampled immediately (time = t_0) using a needle punched through each stop-

per. The oxygen tension (P_{O_2}) at t_0 was determined using a Radiometer E 5046/0 electrode. At intervals of 5 min thereafter, oxygen tensions were determined in a similar manner. These data were used to determine the rate of decrease of oxygen tension in the water. Following each experiment, the weight of each fish was determined and its volume was measured. The volume of each respiratory chamber was also measured. These values were used to determine the specific rate of oxygen consumption (mg O_2 kg^{-1} h^{-1}) of each trout.

Determination of ventilation volume. Ventilation volume was calculated by the Fick principle (Guyton 1981) using the following equation:

$$V_g = V_{O_2}/(Pi_{O_2}-Pe_{O_2})\ a_{O_2}$$

where V_g = ventilation volume (ml min^{-1} kg^{-1}); V_{O_2} = O_2 uptake (ml min^{-1} kg^{-1}); a_{O_2} = solubility coefficient of oxygen in water (ml ml^{-1} mm Hg); P = partial pressure of gas (mm Hg); and the subscripts i and e refer to water in the buccal cavity (inspired) and opercular cavity (expired) respectively.

Determination of intracellular pH. Red blood cells obtained by centrifuging 600 µl of blood at 20 000 × g for 5 min were frozen to −20 °C, thawed for 5 min and refrozen to −20 °C. To prevent the acid shift observed when lysed samples remain unfrozen, measurements of intracellular pH (pH_i) were made immediately after the second thawing using a Radiometer G 2971 G2 electrode in a thermostated cuvette and recorded using a Radiometer PHM meter.

Statistical analyses. Values are expressed as the arithemitic mean ± standard error (SE). All comparisons between treated and control fish were made by 1-way analysis of variance.

RESULTS

The mean concentration of *Chaetoceros concavicornis* cells remained between 51 and 58 cells ml^{-1} during the 72 h course of the experiments described below (Fig. 1).

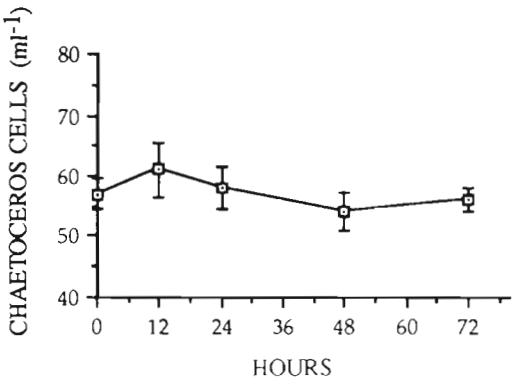

Fig. 1. Concentration of *Chaetoceros concavicornis* cells in the treated seawater

The inter-secondary lamellar spaces of the trout quickly accumulated a compact mass of *Chaetoceros concavicornis* cells (Fig. 2A). And, some of the spines of these diatoms appeared to penetrate the lamellar cells (Fig. 2B). Coincident with this entrapment of diatoms in the inter-secondary lamellar spaces and the apparent penetration of the cells with the barbed spines, there was an accumulation of mucus-like material on the surfaces of the secondary lamellae (Fig. 2C); indeed, in some cases, a mass of *C. concavicornis* and what appeared to be mucus filled the volumes between many adjacent secondary lamellar cells (Fig. 2D). The mucous cells of the secondary lamellae of treated trout were more numerous and more prominent than those of this organ in untreated control trout.

Histological examination of gills of fish treated with *Chaetoceros concavicornis* showed hyperplasia, hypertrophy and partial and complete fusion of secondary lamellae (Fig. 3A). The fish also showed severe edema, collapsed pillar cell systems, detachment of epithelial cells from the walls of the blood capillaries and localized hemorrhaging of some secondary lamellae (Fig. 3B) in comparison to the lamellar cells of untreated fish (Fig. 3C).

The mean P_{O_2} of the blood sampled from the dorsal aorta of the treated fish decreased from ca 100 mm Hg to ca 50 mm Hg over a 72 h period when the fish were treated with water containing between 51 and 58 cells ml^{-1} of *Chaetoceros concavicornis* (Fig. 4). The mean P_{O_2} of blood sampled from the dorsal aorta of the control fish untreated with *C. concavicornis* remained approximately the same over the 72 h period (Fig. 4). The P_{O_2} difference between treated and control fish was significant ($p < 0.001$).

The mean pH of the dorsal aorta blood (pH_a) of fish decreased from 7.81 to 7.64 following *Chaetoceros concavicornis* treatment for 72 h while the mean pH of the untreated control fish remained relatively constant at 7.77 to 7.83 (Fig. 5).

The mean ventilation volume of treated fish increased significantly ($p < 0.001$) to 3666 from 1258 ml kg^{-1} min^{-1} during the 72 h exposure to *Chaetoceros concavicornis*, while the mean ventilation volume of the untreated control fish remained within the range of 1211 to 1298 ml kg^{-1} min^{-1} during this same time period (Fig. 6). The difference is significant.

The mean ventilation frequency of treated fish increased to 131 from 79 times min^{-1} following a 72 h exposure to *Chaetoceros concavicornis* (Fig. 7). The mean ventilation frequency of the control fish remained within the range of 71 to 81 times min^{-1} (Fig. 7). The ventilation frequency difference between treated and control fish was significant ($p < 0.005$).

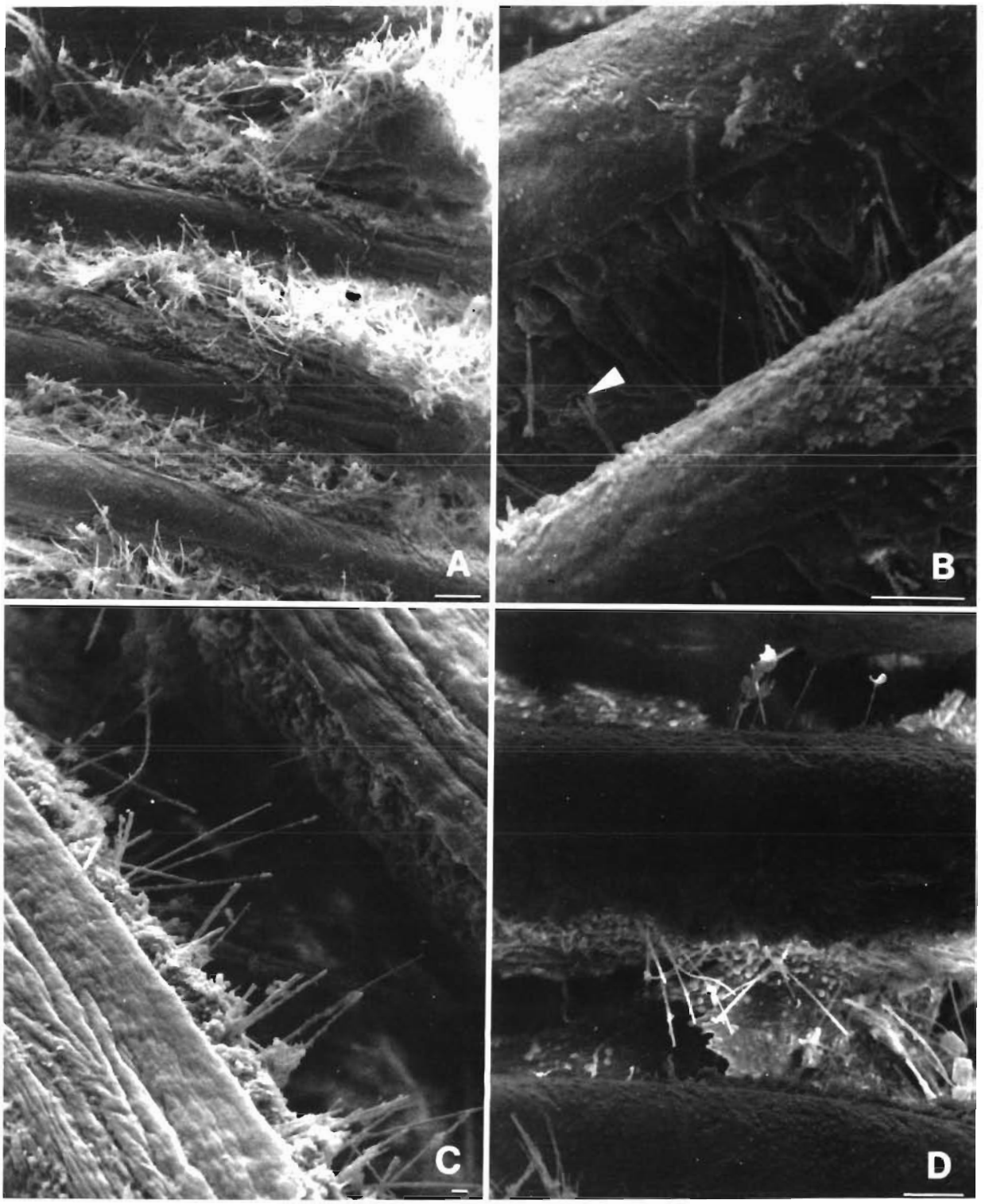

Fig. 2. *Chaetoceros concavicornis* on gill tissue of *Oncorhynchus mykiss*. Scanning electron microscopic views of: (A) *C. concavicornis* between secondary lamellae; (B) penetration of lamellar cells by spines; (C) accumulation of mucus on lamellar cells and (D) inter-secondary lamellar accumulations of *C. concavicornis* cells and mucus-like material. Scale bars are 100 μm in (A), (B) and (D), 10 μm in (C)

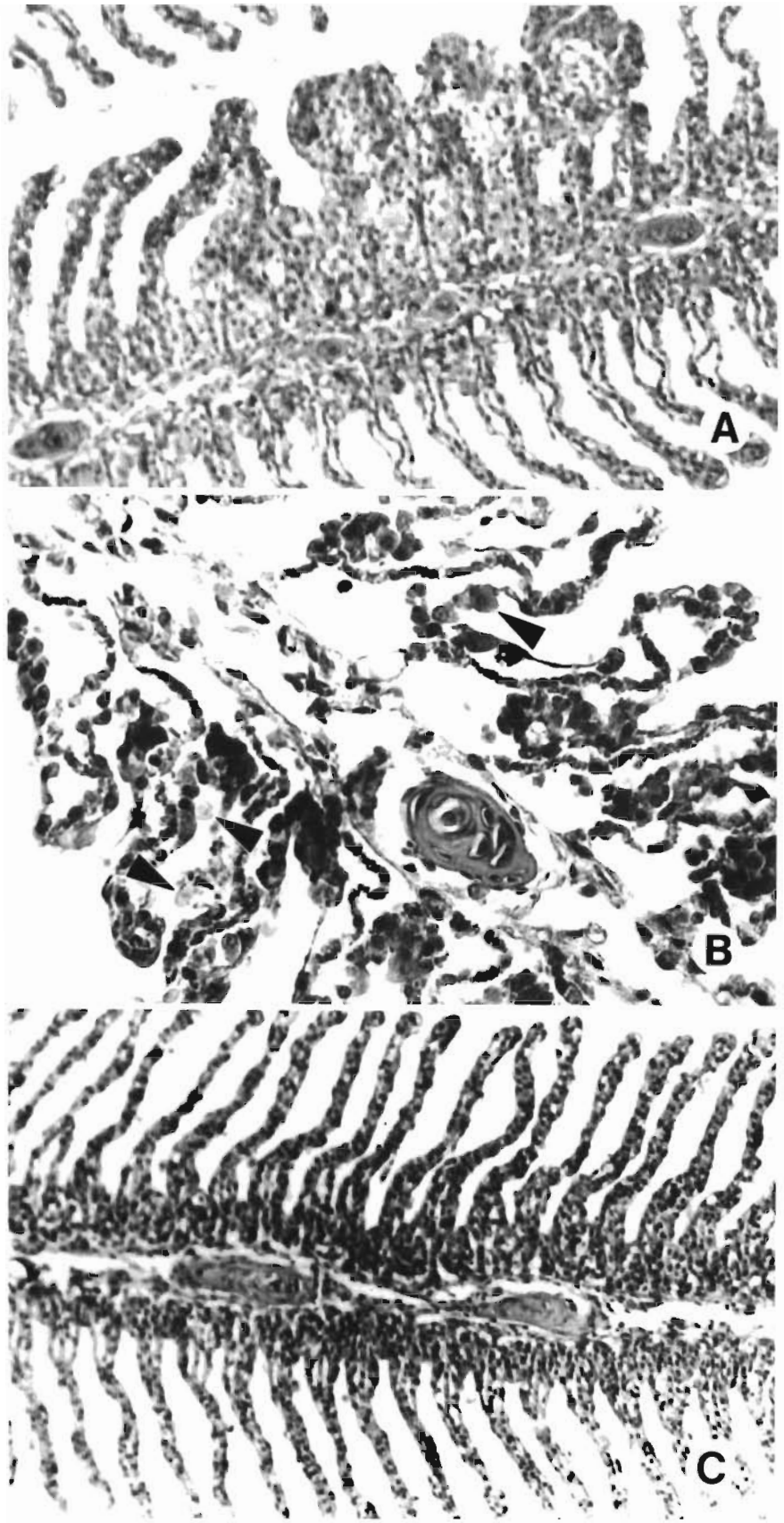

Fig. 3. *Oncorhynchus mykiss* exposed to *Chaetoceros concavicornis*. Light microscopic views of: (A) gill lamellae of *C. concavicornis* treated rainbow trout; note hyperplasia, partial and complete fusion of the secondary lamellae. H & E staining, 150 ×; (B) gill lamellae of *C. concavicornis* treated rainbow trout; note severe edema, collapsed pillar cells, hemorrhaging (arrowhead) and detachment of the secondary lamellae. H & E staining, 240 ×; (C) gill lamellae of untreated rainbow trout. H & E staining, 150 ×

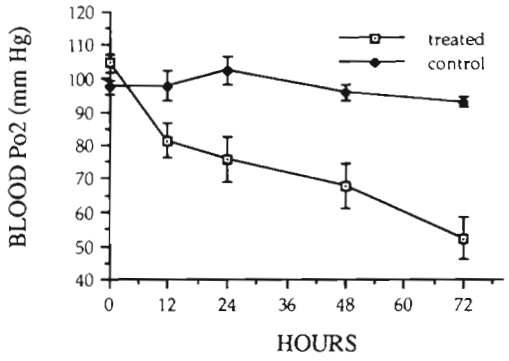

Fig. 4. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Partial pressure of oxygen in the dorsal aorta blood of trout

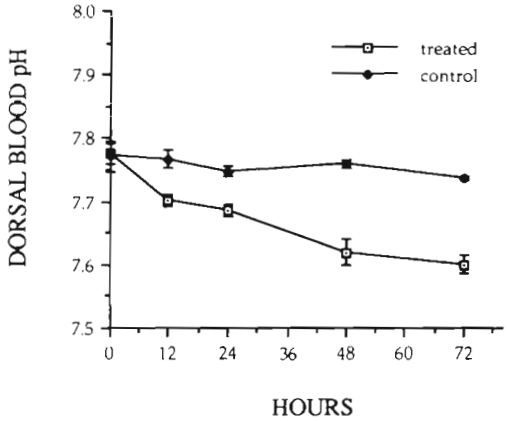

Fig. 5. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Dorsal aorta blood pH_a of trout

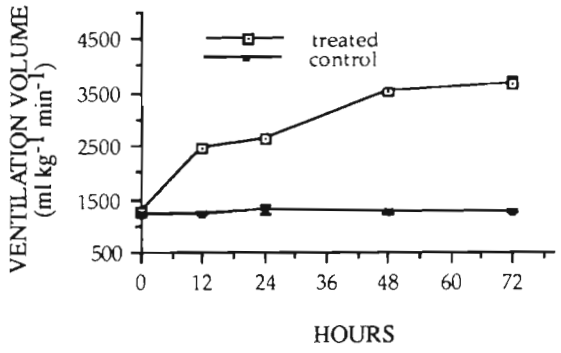

Fig. 6. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Ventilation volume of trout

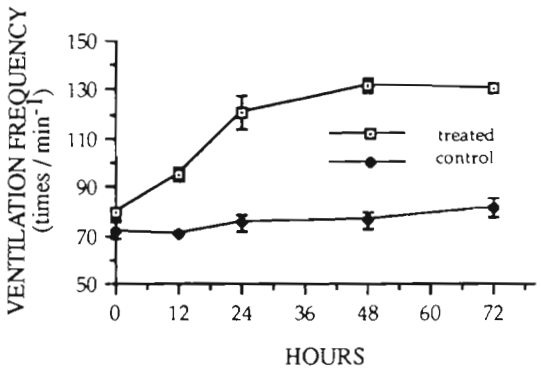

Fig. 7. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Ventilation frequency of trout

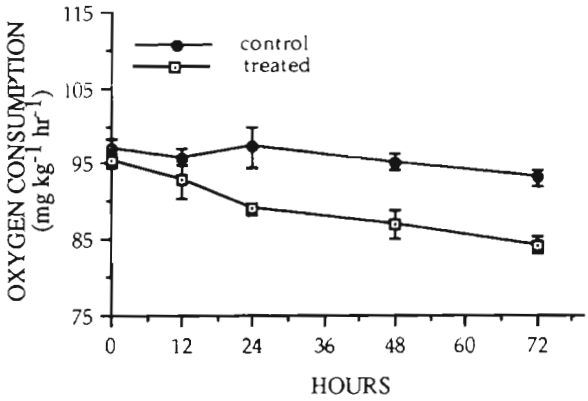

Fig. 8. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Oxygen consumption of trout

The mean O_2 consumption of treated fish decreased from 95 to 88 mg kg^{-1} h^{-1} following a 72 h exposure to *Chaetoceros concavicornis* (Fig. 8). The mean O_2 consumption of the untreated control fish remained within the range 93 to 97 mg kg^{-1} h^{-1} (Fig. 8). There was no significant difference between the O_2 consumption of the treated and untreated fish during the first 12 h (Fig. 8). However, there was a significant difference between the O_2 consumption of the treated and untreated fish following the first 12 h ($p < 0.05$).

Twelve hours after exposure to *Chaetoceros concavicornis* the mean haematocrit value increased to ca 22.1 % from ca 18.9 % (the value of the control fish at 12 h was ca 18.8 % (Fig. 9). There was a significant difference between the values for the treated and untreated trout ($p < 0.001$).

The mean lactate concentration of the blood of the treated trout increased to 36.9 mg 100 ml^{-1} following *Chaetoceros concavicornis* treatment for 72 h in comparison to a value of 25.0 mg 100 ml^{-1} prior to treatment (Fig. 10). The mean concentration of lactate in the blood of the untreated control trout remained approximately the same during the 72 h of the experiment (Fig. 10). There was a significant difference between the lactate concentrations of the blood of treated and untreated trout ($p < 0.005$).

The mean glucose concentration of trout blood increased to 60.9 mg 100 ml^{-1} following a 72 h treatment with *Chaetoceros concavicornis* (Fig. 11); the mean glucose concentration of these trout prior to *C. concavicornis* exposure was 43.6 mg 100 ml^{-1} (Fig. 11).

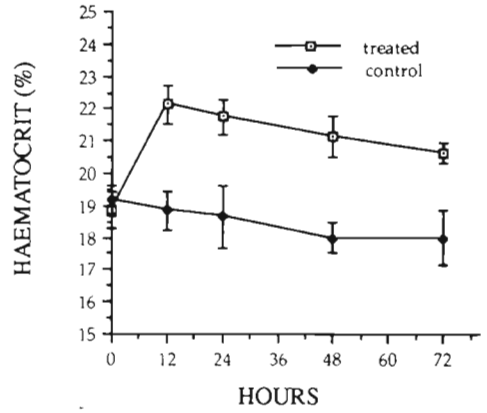

Fig. 9. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Haematocrit of trout

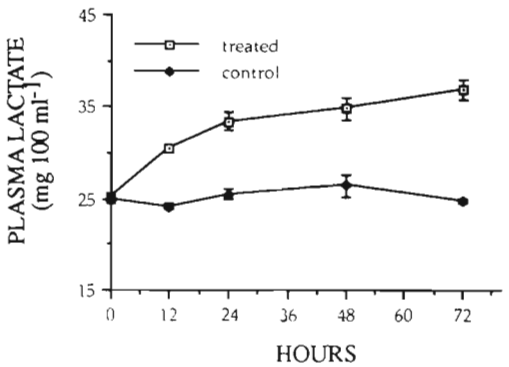

Fig. 10. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Blood lactate concentrations of trout

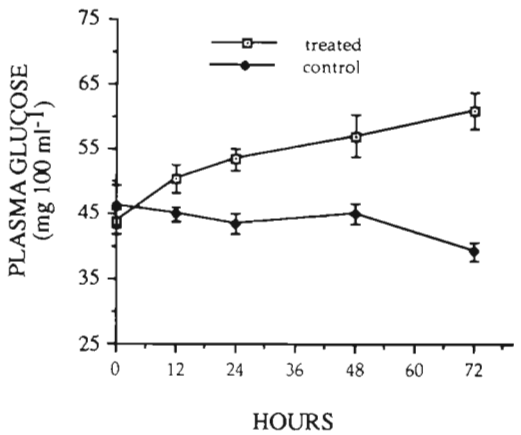

Fig. 11. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Blood glucose concentrations of trout

The mean concentration of blood glucose of the untreated fish remained between 46.2 and 39.3 mg 100 ml^{-1}. There was a significant difference between the blood glucose concentrations of the treated and control trout ($p < 0.005$).

The mean erythrocyte pH_i of the treated fish

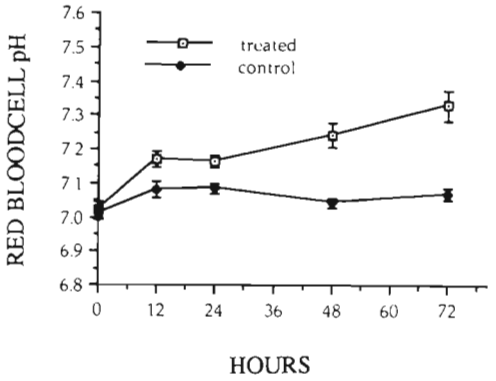

Fig. 12. *Oncorhynchus mykiss* treated with *Chaetoceros concavicornis* cells. Intracellular erythrocyte pH (pH_i) of trout

increased from 7.02 prior to treatment to 7.33 following *Chaetoceros concavicornis* treatment for 72 h (Fig. 12). The mean pH_i values of the control fish varied from 7.01 to 7.09 during the 72 h of the experiment (Fig. 12).

DISCUSSION

Scanning electron micrographs clearly showed that *Chaetoceros concavicornis* cells and their barbed spines become wedged in the inter-secondary lamellar spaces of these fish to form what appears to be 'windrow-like' masses of immobilized diatoms and their broken spines (Fig. 2A). Many of the spines have broken off the diatom frustules and some appear to have penetrated the lamellar cells (Fig. 2B). A careful examination of these micrographs (especially Fig. 2C, D) and those of Fig. 3 indicates that the cells of this tissue appear to respond to this injury in several ways. One of the responses is the formation of what appears to be excessive amounts of mucus on and between the secondary lamellae of the treated fish (Fig. 2C, D). A histological analysis of this tissue further showed that many of these lamellar cells underwent hypertrophy and hyperplasia 72 h after *C. concavicornis* treatment (Fig. 3). These observations cannot be used to determine the sequence of events which occur upon exposure of these trout lamellae to harmful concentrations of *C. concavicornis*. Nevertheless, it would appear likely that the production of mucus would be initiated first, followed by cell hyperplasia. Cell hypertrophy may occur coincident with or following the initiation of mucus production.

We observed a small amount of hemorrhage of secondary lamellae (Fig. 3C) but did not observe any overt signs of secondary infections caused by *Chaetoceros concavicornis* during the 72 h of this experiment, both

of which processes were suggested by Bell (1961) to be mechanisms by which *C. concavicornis* may cause death of affected finfish.

Based upon the above observations, it appears that the production of excess mucus, hypertrophy and hyperplasia of the respiratory epithelium cells of the trout, caused by exposure to this harmful phytoplankter, will physically limit gill functions, including gaseous and waste product exchange at the interface of secondary lamellar cells within the aqueous environment. The reduced oxygen-diffusing capacity of the gills in particular would in turn initiate a cascade of events in the respiratory chain of the trout. One would therefore predict changes to functions such as aortal blood O_2 pressure and pH, ventilation volumes and frequencies, plasma lactate and glucose concentrations, haematocrit and erythrocyte pH_i.

The effects of environmental hypoxia on respiratory gas transport in teleost fishes have been studied extensively, and adaptational responses have been found in the respiratory chain including changes in arterial blood oxygen tension, blood pH, ventilation volume, ventilation frequency and O_2 consumption (Holeton & Randall 1967a, Randall 1982). Specific responses of the various portions of the trout respiratory transport system as they relate to exposure to *Chaetoceros concavicornis* are discussed below.

The continuous depletion of O_2 with time from arterial blood in *Chaetoceros concavicornis* treated fish (Fig. 4) probably is directly caused by diffusion limitation at the secondary lamellae due to excess mucus production. This reduction in arterial O_2 pressure then affects the trout's ventilation volumes and frequencies. Smith & Jones (1982) have shown an effect similar to the one noted herein, where they found that a reduction of blood oxygen pressure by hypoxia in rainbow trout stimulated the animal's ventilation volume. Similar effects have been noted by Randall & Jones (1973) and Holeton & Randall (1967a).

Under hypoxic conditions the fish attempts to maintain its blood O_2 at required concentrations by increasing both its ventilation volumes and frequencies, the actual values varying with the species (Smith & Jones 1982). Holeton & Randall (1967b) found that rainbow trout ventilation frequency increased from ca 80 to ca 120 min^{-1} with advanced hypoxia. In our experiments, although the ventilation frequencies of the control trout varied between 70 and 80 min^{-1}, the *Chaetoceros concavicornis* treated trout had frequencies in excess of 130 min^{-1} (Fig. 7), indicative of acute hypoxia.

The approximately 0.2 pH increase in acidity of the blood within ca 24 h of exposure of the trout to *Chaetoceros concavicornis* could involve several factors, e.g. a change in plasma ionic composition and/or an increase in plasma metabolic acids. Following hypoxia, H^+ is believed to be extruded from erythrocytes, triggered by the release of catecholamines to the circulation which in turn stimulates Na^+/H^+ exchangers located in their membranes. This has been shown *in vitro* for adrenaline-treated (Nikinmaa 1982) and isoproterenol-treated (Nikinmaa & Huestis 1984) erythrocytes of rainbow trout. Nikinmaa (1982) and Cossins & Richardson (1985) suggested that the H^+ extrusion could increase the hemoglobin oxygen affinity via the alkalization of the intracellular pH. The increase of lactate levels in blood reported herein may be associated with the decrease in pH. Tetens & Lykkeboe (1985) reported lower blood pH after fish were exposed to hypoxic conditions. They suggested that the decreased pH in the blood was possibly caused by a release to the circulation of protons from muscle lactate.

Hypoxia of rainbow trout can cause metabolic acidification of the blood (Thomas & Hughes 1982). In addition, the pH_i increase (Fig. 12) and pH_a decrease (Fig. 5) noted in our experiments are consistent with what has been observed in other experiments with teleosts undergoing hypoxia (Thomas & Hughes 1982, Claireaux et al 1983, Fievet et al. 1988).

The very rapid increase in haematocrit values of dorsal aorta blood within 12 h of *Chaetoceros concavicornis* treatment may have been due to division of erythrocytes (Murad et al. 1990) and/or elevation of plasma catecholamine levels of the fish which could induce swelling of erythrocytes, entry of erythrocytes into the circulatory system from storage organs, such as the spleen, and plasma skimming under the influence of hypoxia (Ostroumova 1964, Johansen & Hanson 1967, Stevens 1968, Hughes 1981, Milligan & Wood 1986, Ishimatsu et al 1988). Whatever the reason, the increased haematocrit concentrations observed in the present study were typical of those observed when teleost fish undergo hypoxia.

Lactate and glucose concentrations have been shown by many investigators to be sensitive indicators of hypoxic stress in rainbow trout (e.g. Holeton & Randall 1967a) as well as several other freshwater and marine species (e.g. Hattingh 1967, Yu & Woo 1987). The pattern of lactate and glucose concentration increases noted in the present work (Figs. 10 & 11) indicates that low blood O_2 concentrations likely triggered anaerobic metabolism in the trout treated with *Chaetoceros concavicornis*. Boutillier et al. (1988) noted that when environmental O_2 concentrations fell below 50 mm Hg plasma lactate levels of rainbow trout rose as increased hypoxia boosted the rate of anaerobic metabolism. In the experiments reported here, the concentration of oxygen exceeded 50 mm Hg in the water. However, this gas was unable to pass the secondary lamellar cells at rates commensurate with the maintenance of aerobic

conditions in the arterial blood, allowing the O_2 concentrations to fall far below the concentrations which one would expect with this high concentration of O_2 in the water.

The data presented here clearly indicate that the barbed spines of the harmful diatom *Chaetoceros concavicornis* damage the physical integrity of the respiratory epithelium of rainbow trout, and by extension, that of other salmonids. The trout responds to this physical impairment of gill tissue by producing excessive amounts of mucus. The accumulation of this mucus on and between the secondary lamellae inhibits the gill functions of O_2 diffusion (and probably metabolic waste products release.) The hypoxic conditions which result within the fish then cause a cascade of events, including anaerobic metabolism, as the trout attempts to compensate for loss of arterial blood O_2. In the experiments described here, impairment of trout respiration was not sufficiently great to cause mortality of the fish within the 72 h of the *C. concavicornis* treatment. However, most of the parameters assayed showed significant increases (or decreases) with time. Had *C. concavicornis* treatment of the fish proceeded beyond 72 h, mortality of the fish would likely have occurred due to this harmful diatom.

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